DEVELOPMENT OF NEW ANALYTICAL METHODS INVOLVING NOVEL SORBENTS AND MEMBRANES FOR THE DETERMINATION OF SELECTED MYCOTOXINS AND RELATED METABOLITES IN BIOLOGICAL FLUIDS AND FOOD

by

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AF	Aflatoxins
ASE	Accelerated solvent extraction
C18	Octadecysilane
CTN	Citrinin
DLLME	Dispersive liquid–liquid microextraction
DON	Deoxynivalenol
DVB	Divinylbenzene
EDX	Energy-dispersive X-ray spectrometer
E_e	Extraction efficiency
E_f	Enrichment factor
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
λ_{ex}	Excitation wavelength
λ_{ex}	Emission wavelength
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FD	Fluorescence

FTIR	Fourier transform infrared
FUMs	Fumonisins
G	Gram
GC	Gas chromatography
HF-LPME	Hollow fiber liquid phase microextraction
HNA	1-hydroxyl 2-naphthoic acid
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography tandem mass spectrometry
IAC	Immunoaffinity columns
IARC	International Agency for Research on Cancer
IL	Ionic liquid
kg	Kilogram
L	Liter
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid- liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LTL	Zeolite Linde Type L
OTA	Ochratoxin A
PAT	Patulin
PES	Polyethersulfone
pKa	Acid dissociation constant
PLE	Pressurised liquid extraction
ppb	parts per billion

MeOH	Methanol
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
µg	Micro gram
µL	Micro liter
µmol	Micro mol
µ-SPE	Micro-solid phase extraction
mg	Milligram
min	Minute
MIP	Molecularly imprinted polymer
mL	Milliliter
MON	Moniliformin
NA	1-Naphthol
ng	Nanogram
NIP	Non-imprinted polymer
NMP	*N*-methyl-2-pyrrolidone
R^2	Correlation of determination
rpm	Revolutions per minute
RSD	Relative standard deviation
Sa	Sphinganine
SD	Standard deviation
SEM	Scanning electron microscopy
SFE	Supercritical fluid extraction
So	Sphingosine
SPE	Solid phase extraction
SPME	Solid phase microextraction
TLC	Thin Layer Chromatography

wt	weight
w/v	weight per volume
XRD	X-ray diffraction
ZEA	Zearalenone

PEMBANGUNAN KAEDAH ANALISIS BARU MELIBATKAN PENJERAP DAN MEMBRAN NOVEL BAGI PENENTUAN BEBERAPA MIKOTOKSIN DAN METABOLIT BERKAITAN DALAM CECAIR BIOLOGI DAN MAKANAN

ABSTRAK

Tesis ini berfokus terhadap pembangunan dan pengesahan kaedah penyediaan sampel secara baru untuk penentuan mikotoksin dan bahan-bahan yang berkaitan. Tambahan pula, suatu kaedah kromatografi cecair prestasi tinggi (HPLC) bagi penentuan spinganin (Sa) dan spingosin (So) dalam sampel air kencing dengan menggunakan turus silika monolitik diterangkan. Dalam keadaan optimum, pemisahan telah dicapai dengan menggunakan campuran metanol:air (93:7, v/v), suhu turus pada 30 °C, kadar aliran 1 mL min^{-1} dan isipadu suntikan sebanyak 10 μL. Pengurangan yang ketara dalam masa pemisahan (kurang daripada 6 min) telah didapati berbanding dengan turus zarah C18 yang memerlukan masa lebih kurang 14 min. Dua puluh dua sampel air kencing dari penderma yang sihat dan tujuh sampel air kencing dari pesakit kanser hati dianalisis menggunakan kaedah ini. Polimer molekul dicetak (MIPs) untuk sitrinin (CTN) telah disediakan dengan asid 1-hidroksi-2-naftoik (HNA) dan 1-naftol (NA) sebagai template tiruan. Seterusnya, membran polimer molekul dicetak hibrid telah disediakan dengan memerangkap zarah NA-MIP ke dalam perancah polietersulfon dengan menggunakan teknik fasa penyongsangan. Membran tersebut telah dinilai bagi pengekstrakan CTN dan telah ditentukan menggunakan HPLC dengan pengesanan pendarfluor. Peroleh semula

CTN yang dipaku ke dalam beras pada 5, 25 dan 100 ng g^{-1} adalah 89.7-94.2%. Teknik penyediaan sampel yang lain menggunakan pengekstrakan fasa pepejal mikro (μ-SPE) yang diisi dengan MIP atau zeolit jenis Lind L (LTL) bagi penentuan okratoksin A (OTA) telah berjaya dikembangkan. Selepas pengekstrakan, analit telah dinyahjerap menggunakan ultrasonikasi dan dianalisis menggunakan HPLC. Dengan menggunakan MIP, pemulihan OTA yang dipaku ke dalam kopi pada 1, 25 dan 50 ng g^{-1}, jus anggur dan air kencing pada 1, 25 dan 50 ng mL^{-1} berjulat 90.6-102%. Kaedah yang dicadangkan telah digunakan bagi analisis tiga puluh lapan sampel kopi, jus anggur dan air kencing. Kehadiran OTA telah dijumpai dalam dalam lapan belas sampel. Walau bagaimanapun, tahap yang ditemui, adalah di bawah had undang-undang. Zeolit LTL dengan panjang, diameter dan saiz zarah yang berbeza (LTL saiz nano, LTL rod, LTL silinder dan LTL jarum) telah disintesis, dicirikan dan digunakan sebagai pengerap dalam bentuk μ-SPE. Pemulihan OTA yang dipaku ke dalam kopi dan bijirin pada 0.5, 10 dan 25 ng g^{-1} adalah 91.7-101%. Kaedah yang dicadangkan telah digunakan terhadap empat puluh lima sampel kopi dan bijirin. OTA berjulat 0.28-9.33 ng g^{-1} didapati dalam dua puluh lima sampel.

DEVELOPMENT OF NEW ANALYTICAL METHODS INVOLVING NOVEL SORBENTS AND MEMBRANES FOR THE DETERMINATION OF SELECTED MYCOTOXINS AND RELATED METABOLITES IN BIOLOGICAL FLUIDS AND FOOD

ABSTRACT

This thesis focuses on the development and validation of new sample preparation methods applied to the determination of mycotoxins and their related substances. Additionally, a high performance liquid chromatography (HPLC) method for the determination of sphinganine (Sa) and sphingosine (So) in urine samples by employing a silica based monolithic column is described. Under the optimized conditions, separation was achieved using a mixture of methanol:water (93:7, v/v), column temperature at 30°C, flow rate of 1 mL min^{-1} and injection volume of 10 μL. A marked reduction in separation time (less than 6 min) was found compared to approximately 14 min by using a conventional C18 particle column. Twenty two urine samples from the healthy donors and seven samples from the liver cancer patients were analyzed using the method. Molecularly imprinted polymers (MIPs) for citrinin (CTN) were prepared with 1-hydroxy-2-naphthoic acid (HNA) and 1-naphthol (NA) as mimic template. Hybrid MIP membrane were next prepared by embedding the NA-MIP particles into the polyethersulfone scaffold using phase inversion technique. The resultant membranes were evaluated for the extraction of CTN and were determined using HPLC with fluorescence detection. The recoveries of CTN of rice spiked at 5, 25 and 100 ng g^{-1} ranged from 89.7-94.2%. Another sample preparation technique employing micro-solid phase extraction (μ-SPE)

loaded with either molecularly imprinted polymer (MIP) or zeolite Linde type L (LTL) for the determination of ochratoxin A (OTA) were successfully developed. After the extraction, the analyte was desorbed using ultrasonication and were analyzed using HPLC. Using MIP, the recoveries of OTA from coffee spiked at 1, 25 and 50 ng g^{-1}, grape juice and urine samples at 1, 25 and 50 ng mL^{-1} ranged from 90.6-102%. The proposed method was applied to thirty-eight samples of coffee, grape juice and urine. The presence of OTA was found in eighteen samples. The levels found, however, were all below the legal limits. Different length, diameter and particle size of LTL zeolite (nanosized LTL, rod LTL, cylinder LTL and needle LTL) were synthesized, characterized and were used as sorbent in the µ-SPE format. The recoveries of OTA of coffee and cereal spiked at 0.5, 10 and 25 ng g^{-1} ranged from 91.7-101%. The proposed method was applied to fourty-five samples of coffee and cereal. OTA ranging from 0.28-9.33 ng g^{-1} was found in twenty-five samples.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to mycotoxins

For decades, the global contamination of food and feed with mycotoxins is a significant problem. Mycotoxins, which originate from the Greek word "*mykes*" meaning mould and "toxicum" meaning poison, are secondary metabolites produced by fungi that are potentially hazardous to humans, animals and crops that result in illness and considerable economic losses (Turner *et al*., 2009). They can be produced either pre-harvest or post-harvest, during storage, transport and processing. According to the Food and Agriculture Organization of the United Nations, 25% of the world grain supply is contaminated with mycotoxins each year (CAST, 2003). Detecting agricultural commodities contaminated with mycotoxins has been an important issue since the discovery of aflatoxin in England in the early 1960s when more than 10,000 turkeys and ducks died within a few months (Kensler *et al*., 2011).

Generally, mycotoxins are of low molecular weight (~700) and they are chemically and structurally diverse. More than 400 mycotoxins are produced by about 350 species of filamentous fungi but only 30 are of much concerned. Of the wide array of mycotoxins, there are a few that are known to impact animal performance and health which include the aflatoxins (AF), ochratoxin A (OTA), trichothecenes, fumonisins (FUMs), fusaric acid, zearalenone (ZEA), and moniliformin (MON). *Aspergillus*, *Penicilium* and *Fusarium* are the three major genera of mycotoxin producing fungi (e.g., deoxynivalenol (DON) and T-2 produced

by *Fusarium* during pre-harvest, OTA produced by (*Aspergillus* and *Penicilium*) and AF produced by *Aspergillus* during post-harvest.

Mycotoxins occur particularly in regions or countries with climates of high temperature and humidity or where there are poor crop harvesting and storage conditions, which encourage mould growth and mycotoxin development (Patterson and Lima, 2010). Major food commodities affected are cereals, nuts, coffee, spices, beans and fruits such as apples, grapes and apricots. They may also be found in beer and wine that result from the use of contaminated barleys and grapes during their productions (Table 1.1). Exposure of mycotoxins to humans and animals is mostly by ingestion of food products prepared from these commodities. They can also enter the human food chain via meat or other animal products such as eggs, milk and cheese as the result of livestocks eating contaminated feed (Figure 1.1). Mycotoxins are stable even in high temperature treatment, therefore food preparation procedures cannot be expected to remove mycotoxins safely (Sherif *et al*., 2009).

Mycotoxins have become a concern in animal production as they may result in economical loss through clinically obscure changes in growth, production and immunosuppression (Bryden, 2012). The United States Food and Drug Administration (FDA) has estimated the annual cost of crop losses due to all mycotoxins can total $932 million, with research and monitoring adding another $500 million to $1.5 billion (Marasas *et al*., 2008).

Table 1.1 Major food borne mycotoxins or mycotoxin groups, their main producing fungal species and the commodities most frequently contaminated (Köppen *et al*., 2010; Li *et al*., 2012).

Mycotoxins	Fungal species	Food Commodities
Aflatoxins (AFs) (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2)	*Aspergillus flavus, A. nomius, A. parasiticus, A. arachidicola, A. bombycis, A. pseudotamarii, A. minisclerotigenes, A. rambellii, A. ochraceoroseus Emericella astellata, E. venezuelensis, E. olivicola*	Maize, wheat, rice, spices, sorghum, ground nuts, tree nuts, almonds, milk, oilseeds, dried fruits, cheese, spices, eggs, meat
Fumonisins (FUMs) (FB1, FB2, FB3)	*Alternaria alternata, Fusarium anthophilum, F. moniliforme, F. dlamini, F. napiforme, F. proliferatum, F. nygamai, F. verticillioides*	Maize, maize based products, corn based products, sorghum, asparagus, rice, milk
Ochratoxins (OTA, OTB, OTC)	*A. alutaceus, A. alliaceus, A. auricomus, A. glaucus, A. niger, A. carbonarius, A. melleus, A. albertensis, A. citricus, A. flocculosus, A. fonsecaeus, A. lanosus, A. ochraceus, A. ostianus, A. petrakii, A. pseudoelegans, A. roseoglobulosus, A. sclerotiorum, A. steynii, A. sulphureus, A. westerdijkiae, Neopetromyces muricatus, Penicillium viridicatum, P. verrucosum, P. cyclopium, P. carbonarius*	Cereals, dried vine fruit, wine, coffee, oats, spices, rye, raisins, grape juice
Type A trichothecenes (T-2 and HT-2 toxin, diacetoxyscirpenol, neosolaniol)	*Fusarium sporotrichioides, F. poae, F. acuminatum, F. culmorum, F. equiseti, F. graminearum, F. moniliforme, F. myrothecium, Cephalosporium sp., Myrothecium sp., Trichoderma sp., Trichothecium sp., Phomopsis sp., Stachybotrys sp., Verticimonosporium sp.*	Cereals, cereal based products

Table 1.1 Major food borne mycotoxins or mycotoxin groups, their main producing fungal species and the commodities most frequently contaminated (continue).

Mycotoxins	Fungal species	Food Commodities
Type B trichothecenes (nivalenol, deoxynivalenol, 3-acetylDON, 15-acetylDON, fusarenon X)	*F. graminearum, F. culmorum, F. sporotrichioides, F. cerealis, F. lunulosporum*	Cereals, cereal based products
ZEA	*F. graminearum, F. culmorum, F. crookwellense, F.equiseti, F. sporotrichioides*	Barley, oats, wheat rice, sesame, soy beans
Patulin (PAT)	*A. clavatus, A. longivesica, A. terreus, P. expansum, P. griseofulvum, Byssochlamys sp.*	Apples, apple juice, cherries, cereal grains, grapes, pears, bilberries
Citrinin (CTN)	*Aspergillus niveus, A. ochraceus, A. oryzae, A. terreus, Monascus ruber, M. purpureus, Penicillium citrinum, P. camemberti*	corn, barley, wheat, apple, red yeast rice
Ergot alkaloids (Ergocornine/inine, Ergocristine/ inine, Ergocryptine/inine, Ergosine/ inine, Ergotamine/inine)	*Claviceps africanana, C. purpurea, C. fusiformis, C. paspali, Neotyphodium coenophialum*	Wheat, rye, hay, barley, millet, oats, sorghum, triticale

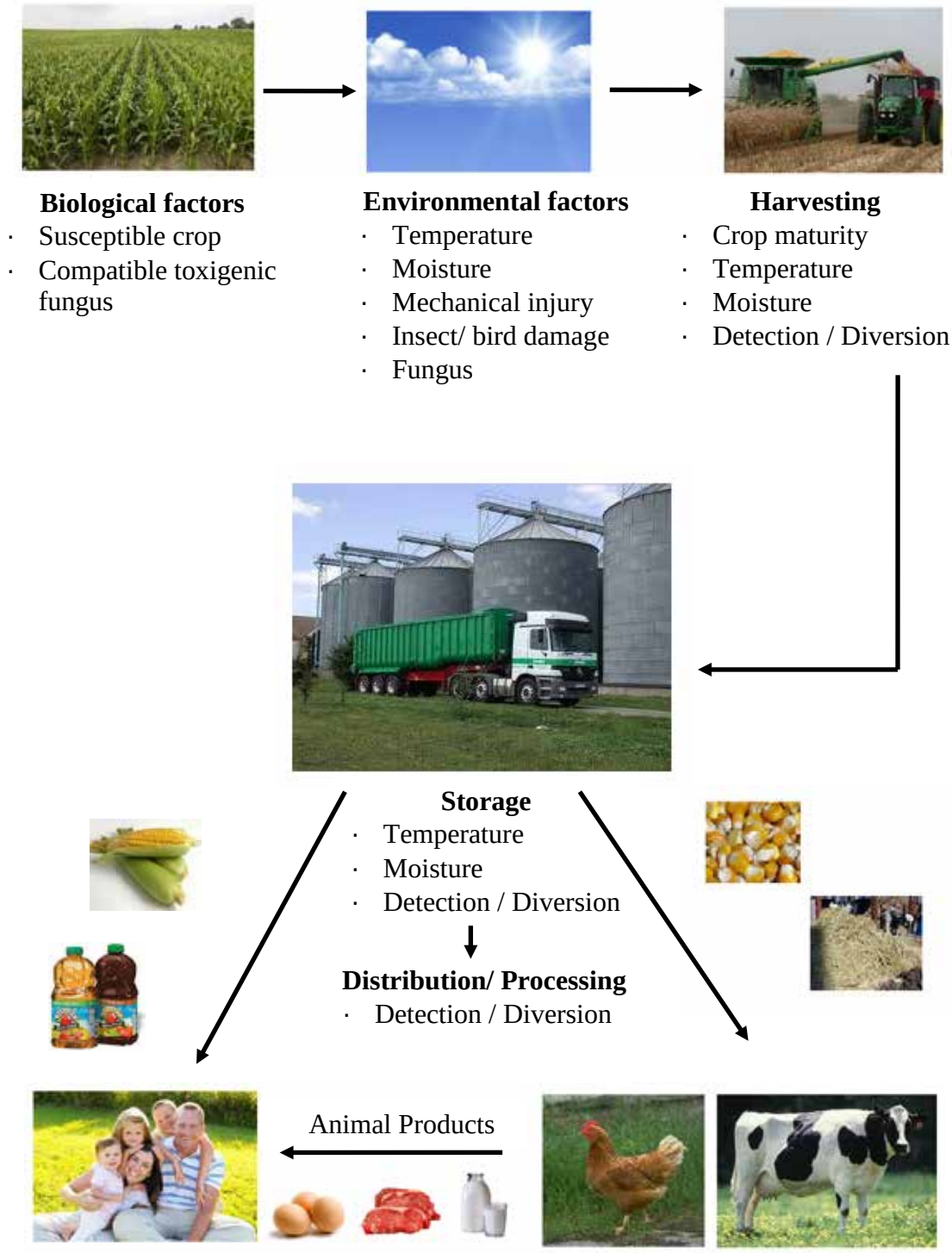

Figure 1.1 Factors affecting mycotoxin occurrence in the human food and animal feed chains.

1.2 Effects on humans and animals health

Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins. The impact of mycotoxins on health depends on the type and amount of the mycotoxin consumed and the toxicity of the compound, the body weight of the individual, the presence of other mycotoxins and other dietary effects (Steyn, 1995). When the levels is sufficiently high, these fungal metabolites can have toxic effects that range from acute (e.g., liver and kidney deterioration), to chronic (e.g., liver cancer), mutagenic and teratogenic, and resulting symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity, and death. The many interacting factors in the pathogenesis of a mycotoxicosis (Figure 1.2) make diagnosis difficult as does confirming mycotoxin exposure (Bryden, 2007).

Ergotism is the oldest identified mycotoxicosis in humans where Ergot is responsible for a disease of the Middle Ages known as “St.Anthony’s Fire” so named for the burning sensation caused in victims’ limbs. This disease is attributed to the human consumption of foods prepared from ergot-contaminated grain. In 1940s and 1950s, another example of mycotoxicosis in humans in Russia occurred, “Alimentary Toxic Aleukia” and was responsible for the death of thousands of people. This devastating disease is known to cause fever, bleeding from the lethal disease skin, nose, throat and gums, necrosis, and suppression of the immune system and mortality reaching 80%, was recognised as toxic manifestation of mold contamination of harvested grains. It has been found that this disease was caused by T-2. AFs were discovered in 1960 following the deaths of 100,000 young turkeys in England,

(Richard, 2007). Some important mycotoxins with their possible health effects are shown in Table 1.2.

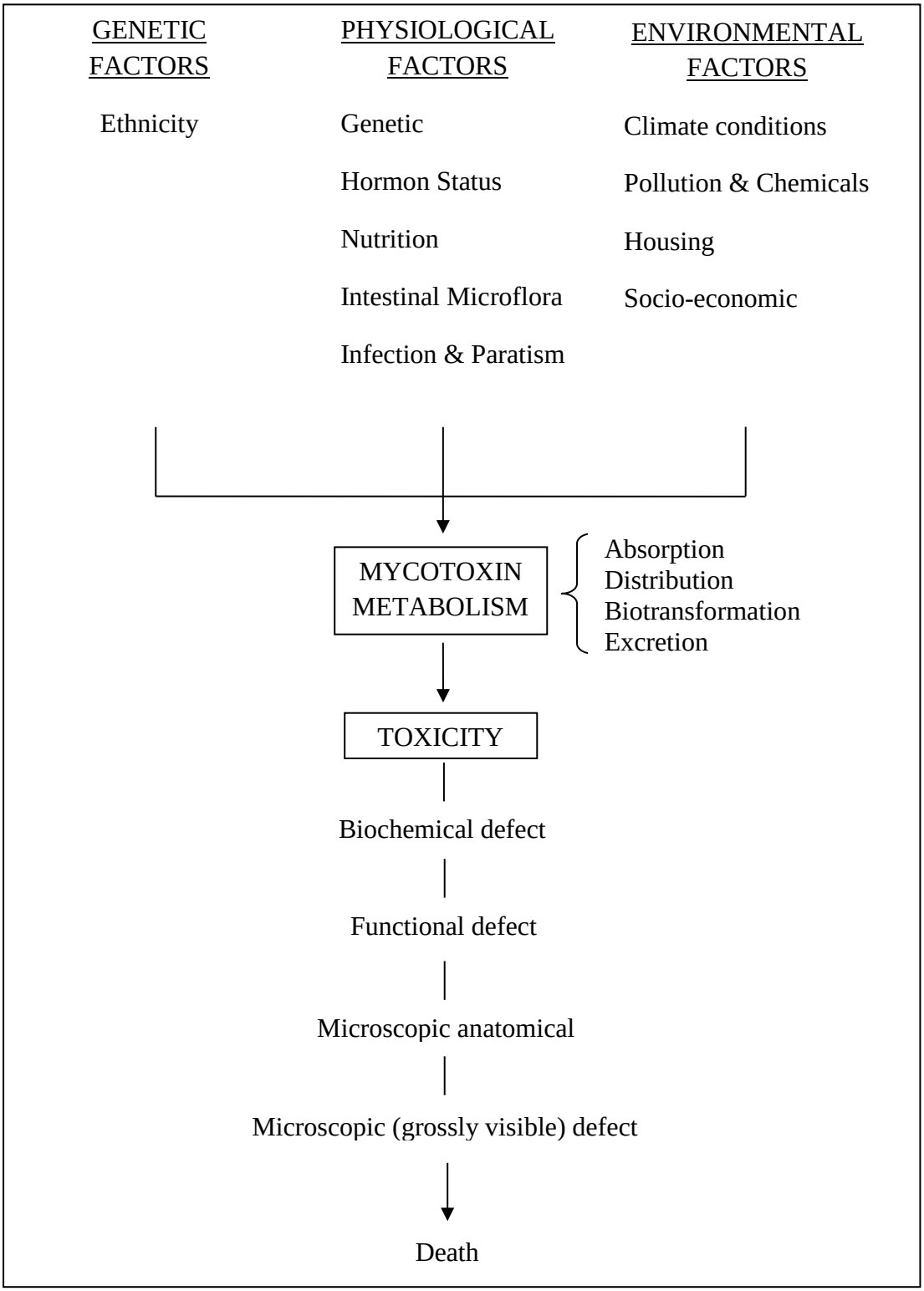

Figure 1.2 A simplified representation of some general relationships in a mycotoxicosis (Bryden, 2007)

Table 1.2 Mycotoxins with their possible health effects (Richard, 2007; Kumar *et al*., 2008)

Mycotoxins	Effects on animals and humans health
AFs	· Classified as Group 1 by International Agency for Research on Cancer (IARC) · Hepatotoxic or cause liver damage in animals · Decreased production (milk, eggs, weight gains, etc.), are immunosuppressive, carcinogenic, teratogenic and mutagenic
FUMs	· Classified as Group 2B by IARC · Oesophageal cancer in regions of Transkei (South Africa), China and northeast Italy · Leukoencephalomalacia (hole in the head syndrome in horses · Lung edema in swine · Liver and kidney tumours in rodents · Hepatotoxic and carcinogenic effects in rats
OTA	· Classified as Class 2B by IARC · Balkan Endemic Nephropathy a kidney disease with associated tumors and urinary tract urothelial cancer in humans · Liver toxin, an immune suppressant, a potent teratogen, and a carcinogen in animals
Trichothecenes	· Weight loss or poor weight gains, bloody diarrhea and decreased production of milk and eggs in animals
DON	· Immunosuppressive and may cause kidney problems in animals · Vomit syndrome in humans
ZEA	· Classified as Group 3 by IARC · Infertility, abortion or other breeding problems in animals
PAT	· Classified as Group 3 by IARC · Mutagenic, genotoxic, carcinogenic.
Ergot	· Gangrene, central nervous system and gastrointestinal effects in humans

1.3 International regulations

The awareness that mycotoxins can have serious effects on humans and animals has led many countries to establish regulations on mycotoxins in food and feed in the last decades to safeguard the health of humans, as well as the economical interests of producers and traders. In most countries specific maximum limits for several contaminants for different foods and a reference to the sampling methods and performance criteria of analysis are recommended. By the end of 2003, approximately 100 countries had developed specific limits for mycotoxins in foodstuffs and feedstuffs, and the number continues to grow.

Regulations are primarily made on the basis of known toxic effects. For the mycotoxins currently considered most significant are AF, FUMs, OTA, DON, ZEA and PAT in different foodstuffs. The Joint Expert Committee on Food Additives (JECFA) acts as a scientific advisory body of World Health Organization and the Food and Agriculture Organization. JECFA provides assessment for the toxicity of additives, veterinary drug residues and contaminants. The evaluation data carried out by JECFA normally results in the estimation of a Provisional Tolerable Weekly Intake or a Provisional Tolerable Daily Intake. In the European Union (EU), efforts to assess exposure are undertaken within the Scientific Cooperation on Questions relating to Food (SCOOP) projects, funded by the European Commission. The SCOOP projects are to estimate intake of several mycotoxins by EU inhabitants. The legal limit of some important mycotoxins within the EU are shown in Table 1.3.

Table 1.3 Legal limit range of mycotoxins according to the Commission regulation (EC) (EC 2006, EC 2007).

Mycotoxins	Legal limit range (μg Kg^{-1})
AFs AFB1 AFM1	4.0 (Dried fruit and nuts) – 15.0 (Groundnuts) 0.1 **(**Dietary**,** processed cereal-based and infants foods) – 8.0 (Groundnuts) 0.025(Infant and dietary foods) – 0.05 (Milk)
Sum of FB1 and FB2	200 (processed maixed based foods) – 4000 (unprocessed maize)
OTA	0.5 (processed cereal-based and infant foods) – 10 (grape juice and instant coffee)
DON	200 (processed cereal-based and maize based infant food) – 1, 750 (Unprocessed wheat and oats, unprocessed maize)
ZEA	20 (processed cereal-based and maize based infant food) – 350 (Unprocessed maize)
PAT	10 (Apple juice and solid apple products and baby foods) – 50 (Spirit drinks and fruit juice)

1.4 Analysis of mycotoxins

Since the discovery of AFs in the 1960s, analytical methods for mycotoxins, which typically occur in μg Kg^{-1} (ppb) range, have been developed. The determination of mycotoxins is essential to minimize the consumption of contaminated food and feed, for monitoring domestic and import surveillance programs, controlling quality of products, establishing regulatory standards and guidelines, validating decontamination procedures, and preparing standard materials for the use in toxicological studies (Trucksess and Pohland, 2002).

The analytical procedure for the determination of mycotoxins in feeds and foodstuffs generally consist of several major steps as shown in Figure 1.3.

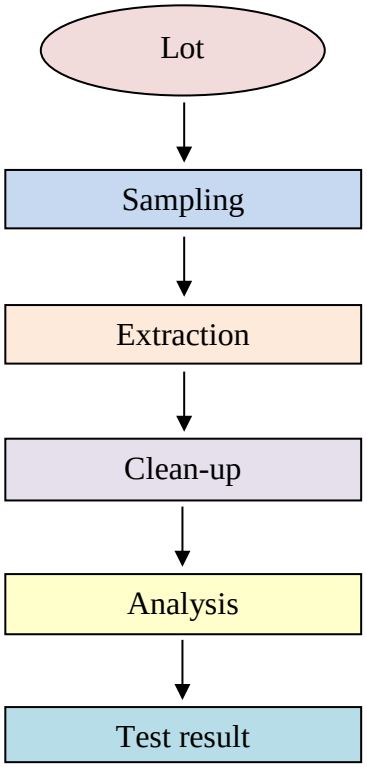

Figure 1.3: General steps involved in sampling, sample preparation, and analysis of mycotoxins in agricultural commodities.

1.4.1 Sampling

Sampling is an important consideration in any monitoring activity. As mycotoxins are not produced homogeneously in the crop, but are the results of fungal growth at specific units, such as maize kernels, wheat kernels, groundnut pods, etc. A single lot of the product will contain hot spots of contamination (Shephard, 2008). The proper selection of a sample from the lot under study and the subsequent steps undertaken to produce a portion for analysis is crucial for the production of sound analytical data. Figure 1.4 shows the general steps of the sampling and analysis

process for whole grain. A lot (an identifiable quantity delivered at one time and assumed to have common characteristics) is sub-sampled to obtain incremental samples and the incremental samples are combined to produce an aggregate sample. The laboratory sample is the sample intended for the laboratory and may consist of the entire, or a portion, of the aggregate sample. The laboratory sample is comminuted, and a test portion is taken from the comminuted laboratory sample. The test portion is defined as the portion of sample taken through the chemical test procedure (Tittlemier *et al*., 2011).

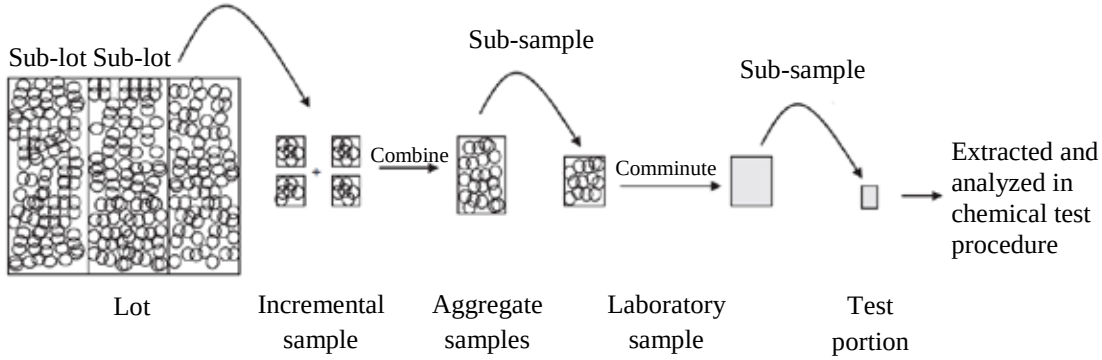

Figure 1.4 Flow diagram of the various steps in the sampling and analysis process for cereal grains (Tittlemier *et al*., 2011).

1.4.2 Conventional methods for the determination of mycotoxins

1.4.2.1 Extraction

As mycotoxins are polar compounds, they are commonly extracted from ground samples by a range of polar solvents or mixtures of solvents. Chloroform ($CHCl_3$) used to be a common solvent for mycotoxins extraction in the early days. However, due to cost and the environmental implications of chlorinated solvents, methanol (MeOH), acetonitrile (ACN) or acetone have been used (Shephard, 2008).

These solvents are mixed with a given ratio of a more polar solvent (e.g., water, dilute acid, aqueous solution of salts) to aid the breaking of weak electrostatic bonds which bind some mycotoxins to other substrate molecules (e.g., proteins) (Meister, 2004, Hartl and Stenzel, 2007). Examples of solvents used in the extraction of mycotoxins is shown in Table 1.4.

The extraction is generally achieved by shaking the sample and extractant or by blending with a homogenizer for a shorter period. In order to speed-up and automate the extraction process, accelerated solvent extraction (ASE) also known as pressurised liquid extraction (PLE) has been used (Zinedine *et al.* 2010; Pérez-Torrado *et al.* 2010). However, the ASE apparatus is expensive and the application of this technique in the field of mycotoxin analysis is only limited to a few laboratories.

Supercritical fluid extraction (SFE) (Sheibani and Ghaziaskar, 2009) with supercritical carbon dioxide as an environmental friendly extraction medium has received a lot of attention in the 1990s. SFE uses a supercritical fluid to extract the required compound from the matrix. This works well due to the high solvating power, and density of the solvating liquid. However, there are some inherent disadvantages in these methods. Using SFE, the polar nature of mycotoxins and their poor solubility in carbon dioxide is a major problem which requires the addition of organic solvent modifiers, such as MeOH or ACN. Besides that, problems also arise with respect to extraction times, analytical recoveries and co-extracted impurities. This technique is not suitable for routine analysis due to the high costs and the need for special equipment (Turner, *et al.* 2009).

Table 1.4 Solvents used in the extraction of mycotoxins samples.

Mycotoxins	Solvents	Matrix	Reference
AFM1	Dichloromethane:acetone	Cheese	Manetta *et al*., 2009
Total AFs and AFB1	MeOH:water	Ground red pepper Pistachio nut	Set and Erkmen, 2010
OTA	Toluene:chloridric acid: magnesium chloride	Cereal	Zaied, 2009
	MeOH:sodium bicarbonate 3%	Coffee	Batista *et al*., 2009
FUMS	ACN:water	Corn-based food and feed	Wang *et al*., 2008
	MeOH:water	Dried figs	Karbancioglu-Güler and Heperkan, 2009
ZEA	MeOH:ACN	Cereal flours	Pérez-Torrado *et al*., 2010
	MeOH	Barley	Wu *et al*., 2011
PAT	Ethyl acetate	Apple puree	Janotová *et al*., 2011
	Ethyl acetate:sodium sulphate :sodium hydrogen carbonate	Apple-based-foods	Barreira *et al*., 2010
CTN	Dichloromethane:phosphoric acid	Grain samples	Meister, 2004
	Ethyl acetate:phosphoric acid	Barley, rye and wheat	Hartl and Stenzel, 2007

1.4.2.2 Clean-up

A clean-up step is essential to minimise the interference of the extract especially when chromatographic techniques are used for their determination at trace levels. Liquid- liquid extraction (LLE) has been used for sample clean-up and is still applied for the determination of PAT in apple juice (Gashlan, 2008). LLE involves exploiting the different solubility of the analyte in the aqueous phase and in immiscible organic phase, to selectivity extract the analyte into one solvent leaving the rest of the matrix in the other. The procedure is effective for several toxins and works well in small-scale preparations. However, these techniques are time consuming, non-specific and require large volumes of organic solvents.

Solid phase extraction (SPE) is an alternative clean-up technique and simpler compared to the LLE. SPE involves the physical adsorption of the analyte on the sorbents that was prepacked in the cartridges. The sample is loaded in one solvent, generally under reduced pressure, rinsed, where most of the contaminants are removed without loss of the analyte and finally the complete elution of the analyte from the SPE cartridge (Figure 1.5). Comparing to LLE, they use considerably less solvent and are faster in operation, easily automated and have a high capacity for binding of small molecules. Besides purification, they can also be used to pre-concentrate the sample providing better detection results.

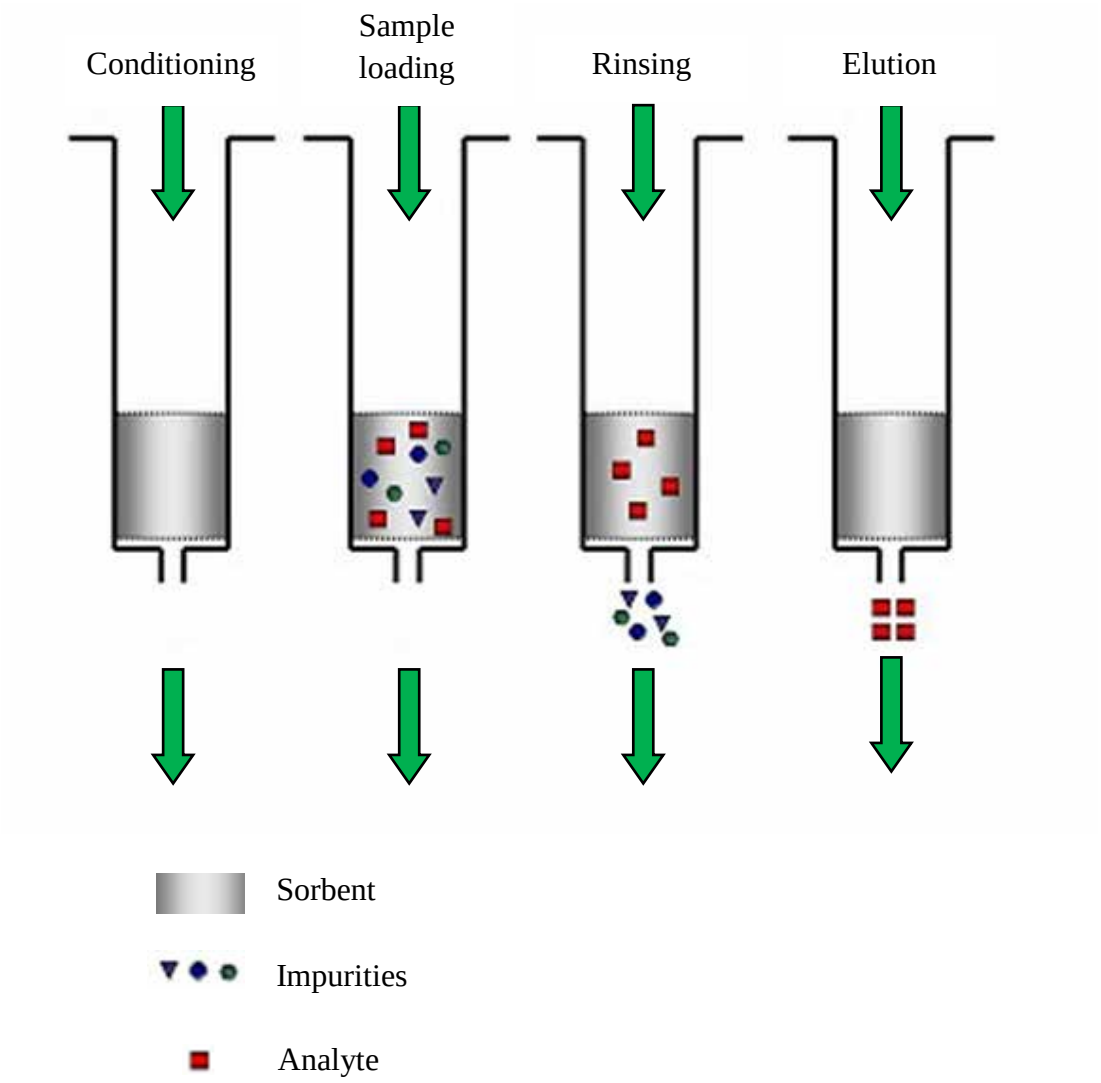

Figure 1.5 Steps in the SPE process

The SPE cartridges contained different sorbents such as silica gel, or florisil, octadecysilane (C18), aminopropyl or ion-exchange material. Ion exchange resin comprise strong or weak anion or cation exchangers bound to a silica support material. Each type can only operate under certain conditions such as pH, solvent used and concentration of the analytes and more importantly, these sorbents relies on relatively non-selective interactions. Thus, sample clean-up with antibody-based immunoaffinity columns (IAC) has become increasingly popular due to its better selectivity. Basically the IAC procedure is based on percolating the sample or the

extract onto a column filled with sorbents of immobilized antibodies against the specific mycotoxin. These columns, however, are rather expensive, of low reusability and exhibit a short lifespan. Table 1.5 shows some SPE methods used in the extraction of mycotoxins.

Clean-up procedures employing multifunctional sorbents, although these are more expensive than the conventional methodologies are also used. Multifunctional columns contain a mixture of charcoal, ion-exchange resins and other materials and are suitable for mycotoxins purification.

Table 1.5 SPE methods used in the extraction of mycotoxins.

Mycotoxin	Sorbent	Matrix	Reference
AFM1	*Hydrophilic Lipophilic Balanced (HLB)*	Liquid milk and milk powder	Wang *et al*., 2010
AFB1	IAC	Animal liver	Tavčar-Kalcher, 2007
OTA	C18, reversed-phase phenylsilane, HLB and IAC	Wine and Beer	Sáez *et al*., 2004
	IAC	Wine	Quintela *et al*., 2011
ZEA	IAC	Corn and corn products	Hewitt *et al*., 2012
PAT	Polyvinylpolypyrrolidone-octadecyl and HLB	Apple juice	Gökmen *et al*., 2005
	C18 SPE	Apple juice	Li *et al*., 2007
CTN	Polyamide	Medicinal and aromatic herbs	Santos *et al*., 2009

1.4.2.3 Analysis

1.4.2.3 (a) Enzyme-linked immunosorbent assay (ELISA)

ELISA has become popular in mycotoxin screening since the late 1970s. In general, this technique involves the reaction of antigen and antibody in micro-plate wells and after adding a chromogenic substrate, allows the quantification of the analyte by means of an optical ELISA reader. ELISA does not require clean-up procedures, and the extract is analyzed directly. Although they often lack sensitivity and limited in the range of matrices examined, immunoassays provide fast, inexpensive screening assays. However, matrix interference or the presence of structurally related mycotoxins can interfere with the binding of conjugate and antibody, leading to mistakes in quantitative measurements of mycotoxins. ELISA kits are recommended be used routinely only for the analysis of matrices that are extensively tested. ELISA kits for FUMs, AFs and DON have been reported recently (Sheng *et al*., 2012 ; Abbès *et al*., 2012; Darleen *et al*., 2012).

1.4.2.3 (b) Thin Layer Chromatography (TLC)

TLC is a simple, cost effective technique, yielding qualitative or semi-quantitative estimations by visual inspection. With densitometric measurements, quantitative results when low detection limits are not required is also possible (Cigić *et al*., 2009). Their advantages include testing a number of samples simultaneously and can also be used as a screening test prior to the more sophisticated instrumental methods. They are also suitable for crude extract analysis, using a wide range of

stationary and mobile phases, as well as an array of spraying agents used for the detection. The majority of TLC analyses of mycotoxins are performed on normal-phase silica gel plates, with the use of two different mobile phases and the development of plates in two different directions giving the greater selectivity.

AFs are naturally fluorescent compounds and they are readily separated by TLC and are easily observed under long wavelength UV light at levels that are useful for quantification of naturally contaminated food samples (Carvalho *et al*., 2012). Quantification is done by visual comparison of the intensities of sample spots of the standards. A number of improvements over the conventional TLC analysis have been introduced and applied to AF analysis such as overpressured-layer chromatography, two-dimensional TLC and high performance TLC (Nawaz *et al*., 1995; Otta *et al*., 2000; Skarkova *et al*., 2000).

For mycotoxins that do not fluoresce, the plate must be sprayed after TLC separation to yield visible spots. Trichothecenes and DON require post-development visualization with spraying reagents: *p*-anisaldehyde, 4-*p*nitrobenzylpyridine (Smoragiewicz *et al*., 1993), and aluminum chloride (Eppley *et al*., 1986) respectively while FUMs have also been determined in corn using reverse-phase TLC plate after spraying with sodium borate buffer, fluorescamine, and boric acid (Rottinghaus *et al*., 1992). An important drawback of TLC methods is that the lack of sensitivity, the detection is sometimes out of the actual legislative limits. Table 1.6 shows the examples of TLC protocols used for detection of common mycotoxins.

Table 1.6 TLC protocols used for the detection of common mycotoxins.

Mycotoxin	Stationary Phase	Mobile Phase	Mode of Detection	Matrix	Reference
AFs	Silica-gel 60G TLC plates	$CHCl_3$:acetone (88:12 , v/v)	Fluorescent	Food products derivable from 'egusi' melon seeds	Bankole *et al*., 2010
CTN	Silica gel H TLC plates	Acetone:ethyl acetate:water (10:10:4, v/v/v)	Fluorescent	Decaying apples	Pepeljnjak *et al*., 2002
	F254 fluorescent silica gel TLC	Toluene:ethyl acetate:formic acid (6:3:1, v/v/v)	UV	Traditional brewed beers	Odhav and Naicker, 2002
	Silica gel 60 TLC plate	Toluene:ethyl acetate:formic acid (5:4:1, v/v/v)	Fluorescent	Fungi isolated from grapes	Abrunhosa *et al*., 2001
FB1	Reverse-phase C18 TLC plates	MeOH:4% aqueous KCl (3:2, v/v)	Fluorescent	Corn	Preis and Vargas, 2000
OTA	Silica gel 60 HPTLC glass plates	Toluene:ethylacetate:formic acid (6:3:1, v/v/v).	UV	Wine	Welke *et al*., 2010
ZEA	Silica gel 60 TLC plate	Ethanol: $CHCl_3$ (3.5 : 96.5, v/v)	UV	Maize samples	Hadiani *et al*., 2003

1.4.2.3 (c) Gas Chromatography (GC)

GC coupled to different detection techniques is widely used in applications for food analysis. They are suitable for thermally stable, non- and semi-polar, volatile and semi-volatile compounds, such as oils and sterols. Most mycotoxins are not volatile and therefore have to be (chemically) derivatised prior to GC analysis. Several techniques have been developed for the derivatisation of mycotoxins. Chemical reactions such as silylation or acylating agents are employed in order to obtain volatile material. For the GC analysis of trichothecenes and other mycotoxins , electron capture detection, flame ionization detector and mass spectrometry (MS) detection have been used (Josephs *et al*. 1998; Labuda *et al*. 2005; Valle-Algarra *et al*., 2011; Nielsen and Thrane, 2011; Ferreira *et al*., 2012).

1.4.2.3 (d) High Performance Liquid Chromatography (HPLC)

HPLC is the most frequently used method, as it is sensitive, have low level of detection and have been developed for almost all the major mycotoxins. Normal and reversed-phase columns are used for the separation and purification of toxins, depending on their polarity. UV or fluorescence (FD) detectors have often been used. Mycotoxins which have natural fluorescence (e.g. OTA, AF, CTN) can be detected directly in HPLC–FD (Köppen *et al*., 2010), but for FUMs that are produced by *Fusarium* genus, which lack suitable chromophore, their determination requires derivatisation.

Pre- or post-column derivatization for FUMs can be performed by using derivatising agents such as are *o*-phthaldialdehyde and naphthalene-2,3-dicarboxyaldehyde (Muscarella *et al*., 2011; Cho *et al*., 2002; He *et al*., 2005). Although AF has natural fluorescence, only B2 and G2 fluoresce with enough intensity in aqueous-based mobile phases to be analytically useful. The enhancement of fluorescence of B1 and G2 can be achieved by pre or post-column derivatization using trifluoroacetic acid or cyclodextrins (Mahoney *et al*., 2010; Mably *et al*., 2005; Cepeda *et al*., 1996).

Several HPLC methods have been adopted as official or standard methods by the Association of Official Analytical Chemists (AOAC) International or the European Standardization Committee (CEN). These methods have been validated, and their analytical characteristics, such as accuracy, repeatability, reproducibility, detection and quantification limits were established. These methods, including the determination of AFs in maize (AOAC Official Method 991.31 and 2005.08), OTA in barley (AOAC Official Method 2000.03), AFB1 in baby food (AOAC Official Method 2000.16), FB1 and FB2 in maize flour and cornflakes (AOAC Official Method 2001.04).

Liquid chromatography-mass spectrometry (LC-MS) methods have recently become more popular despite the high costs and the need for experienced staff. This method offers good sensitivity and has been used for positive confirmation. The limitations of conventional HPLC methods, such as the need to derivatise samples before the analysis have led to the more common use of LC-MS methods. Efforts have been made to develop analytical methods for the simultaneous determination of

different classes of mycotoxins (Table 1.7) using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). This trend is a result of the discovery of the co-occurrence of different toxins. The advantages of this method are the high sample throughput, thus decreases the costs per analysis.

1.4.3 Emerging sample preparation methods for the determination of mycotoxins

Due to the relatively low concentrations of mycotoxins in samples, a suitable sample preparation procedure is required to extract and concentrate the analyte of interest prior to instrumental analysis. Conventionally, LLE and SPE were used, as discussed in section 1.4.2.2.

Increasing attention has recently been devoted to reducing sample volume, analysis time and cost, eliminating the use of (especially chlorinated) solvents and automation, to reduce the workload (Wille *et al*., 2007). In this section, emerging techniques for the determination of mycotoxins such as microextraction methods (e.g., solid-phase micro-extraction, liquid-phase microextraction) which addresses the pertinent issues will be discussed.

1.4.3.1(a) Matrix solid-phase dispersion (MSPD)

MSPD, introduced in 1989, is a simple and cheap sample preparation technique that involves the simultaneous disruption and extraction of various solid, semi-solid and highly viscous materials. It combines sampling, extraction and clean-

Table 1.7 HPLC and LC- MS methods used for the detection of common mycotoxins.

Mycotoxin	Mobile phase	Detector	LOD ($\mu g\ Kg^{-1}$)	RT, min	Matrix	Reference
AFs	MeOH:ACN:water (20:20:60, v/v)	Fluorescence excitation wavelength (λ_{ex}) = 366 nm; emission wavelength (λ_{em})= 440 nm	0.01- 0.17	22	Cereal products	Campone *et al*., 2011
	Solvents A (0.1% formic acid in water) and B (0.1% formic acid in ACN)	Triple-quadruple MS/MS with ESI interface in positive mode	0.003 - 0.007	2.5	Lotus seeds	Liu *et al*., 2012
OTA	5 mM sodium acetate:ACN: MeOH (40:30:30, v/v/v)	Fluorescence (λ_{ex}) = 333 nm; (λ_{em})= 467 nm	0.05	18.7	Peanut samples	Afsah-Hejri *et al*., 2012
FUMS	ACN:water (both acidified with 0.2% formic acid)	Triple–quadrupole mass spectrometer with with an ESI interface in positive mode	10	12.0	Masa, Maize, and Derived Products	Pietri and Bertuzzi, 2102
CTN	0.1 .1% phosphoric acid with water:ACN (55:45, v/v)	Fluorescence (λ_{ex}) = 331 nm; (λ_{em})= 500 nm	0.8	14.0	Red yeast rice, medicinal plants and their related products	Li *et al*., 2011
ZEA	ACN:MeOH:water (45:10:45, v/v/v)	Fluorescence (λ_{ex}) = 274 nm, (λ_{em})= 440 nm	2.0	12.0	Corn and corn products	Hewitt *et al*., 2012